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INDICATIONS RESPECTING THE NATURE OF THE INFECTIVE PARTICLES IN THE MOSAIC DISEASE OF TOBACCO[1]

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Among plant pathologists there is to-day no topic of more engaging interest and no problem more difficult than that of the nature of the causal agency in mosaic and allied plant diseases. Possibly we might extend this statement so as to comprehend at the same time the causal factors involved in those types of vegetative variegation, or modified pigmentation, whether infectious or not, which afford the decorative mottled, spotted, and striped plants so much cultivated for foliage effects.

At various times almost every conceivable view has been held as to the nature of the etiological agent in tobacco mosaic, but from the earliest experiments it has been perfectly clear that the disease is transmissible. The great majority of workers have accepted the evidence of the filterable character of the infective agency, and it is this which gives to the disease much of its peculiar interest.

The relation of the true mosaic diseases to certain other types of

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[1] This paper was read at the annual meeting of the American Philosophical Society, Philadelphia, April 21, 1923.

plant disease involving chlorosis, whether with or without mottling, has not been positively determined, but, for the most part, there are some common characteristics of all to which at least passing reference must be made in the course of this paper. The relation of mosaic diseases to infectious or non-infectious "natural" variegation (yellow and green) of foliage plants also remains for detailed study. In this paper we propose to discuss more particularly some of the problems relating to infectious chlorosis with special emphasis on that type illustrated by the mosaic disease of tobacco.

The true mosaic "diseases" of dicotyledons constitute a somewhat homogeneous group, for they exhibit a blotching or mottling —defined as a mosaic—in which usually both a hypoplastic and a hyperplastic development of the tissues ensues. The mottling is very largely confined to the leaves and may be characterized by regions of lesser chlorophyll development (often a definite yellowing or chlorosis) and regions of intensified chlorophyll development. The latter has sometimes been treated as a quantitative intensification of the chlorophyll and the former as a degradation or diminution of chlorophyll. In discussing the symptomatology of such diseases it has been more the custom to emphasize as the disease the chlorotic areas; and certainly if the sugar cane, maize, and certain other monocotyledonous "mosaics" are included in the category of true mosaics, then the chlorotic areas are admittedly strongly diseased. Abnormal greenness may be, nevertheless, so characteristic that this too should have its place among the symptoms.

Among those who have studied tobacco, bean, and similar mosaics the view has been held also that the intensified green areas are primarily those of disease. In the studies of Dickson ('22) stress is properly laid, it seems to us, upon both aspects of chlorophyll change. Here, as in the earlier important work of Iwanowski ('03), it is clearly shown that in the leaves intensified greenness is correlated with, and in part (at least) due to, increased development of chlorophyllous tissues,—hyperplastic changes. This condition prevails as a part of the differentiation which is produced in young leaves, or in leaves formed after infection. There is no such result in organs already mature at the time of infection. Woods ('00, '02), Chapman ('17), and others have also given attention to the anatomy of mottled areas. The yellow or chlorotic areas are ordinarily correlated with regions of lesser development of the chlorophyllous tissues,—with hypoplastic development. It is of some interest to note that in a typical mosaic disease of swiss chard observed by the senior writer at the Missouri Botanical Garden in 1919 the chief, if not sole, visible color effect was intensified greening in a blotched pattern. In general, there is in dicotyledonous mosaics a focal distribution of effects, and possibly it may be strictly analogous to conditions in human measles, or to the effects produced by an injection into the body of diphtheria antitoxin, or to the focal distribution of pigment in certain skin diseases. In the mosaic disease of tobacco, necrosis, as generally understood in plant pathology, does not occur.

It should not, however, be assumed that marked mottling is necessarily a symptom of these diseases, since many plants or plant species, not themselves seriously mottled, may exhibit, through infection experiments, evidence of severe attacks of the disease so far as this may be expressed through the infectiousness of their juices. Moreover, dwarfing of the general plant, spindling shoots, abscission of blossoms, and many other characteristics are typical of mosaic diseases as they are understood in certain plants, notably in the potato and in certain cucurbits.

Mosaic diseases are recognized to occur in many species of *Solanaceae* (night-shade family), *Cucurbitaceae* (gourds, squashes, etc.), *Leguminoseae* (peas, clovers, etc.), *Chenopodiaceae* (beet and spinach family), *Rosaceae* (raspberries, etc.), *Gramineae* (grasses, sugar-cane, etc.), and many others, altogether about 20 families.

There have been several possible views as to the nature of the causal agency or agencies in mosaic diseases, all or nearly all of which have been exploited, and perhaps very nearly discarded.

ENZYME THEORY

The chief adherent of the enzymic nature of mosaic disease has been Woods ('99, '00, '02). He postulated that the cause of mosaic may be found in an enzyme disturbance in which the chlorotic condition might result from an extensive development of oxidizing enzymes. Unfortunately, this would not explain the condition prevailing where the tissues are hyperplastic, though, if found consistent, it might explain the hypoplastic relation. Moreover, the suggestion that oxidase inhibition upon diastase would explain the accumulation of starch in diseased tissues does not well apply, since the starch accumulation has been shown by Freiberg ('17) and Dickson ('22) to be in the greener areas, a finding confirmed by ourselves. Finally, Allard ('16) has convincingly demonstrated that the infective agency and oxidase are not the same, for a differential and quantitative destruction of the oxidase does not affect infectivity, whereas it is possible also to destroy the active agency in the mosaic disease and yet demonstrate oxidase action. Woods' viewpoint has been adopted also by Heintzel ('00), and in part by Chapman ('13). After criticising (Hunger, '03) the oxidase theory of Woods, Hunger ('05) regards the disease as a nutritional one possessing the peculiar property of being "physiologically autocatalytic," acting by contact and also able to regenerate itself. In some work done in this laboratory Freiberg ('17) also advocated the enzyme viewpoint rather than the more general virus effect, but he considered the enzyme to possess none of the nature of oxidases. The idea that it may be an enzyme was based in part on its absorption by talc, on the specificity of the reaction between the mosaic agency and formaldehyde, and likewise on the basis of the resistance of the body to antiseptics in general. Just how the reproduction of such an enzyme might be accomplished was not considered in detail, but was accounted for on general physiological grounds. In this connection attention was drawn to the fact that upon the injection of the toxin of *Bacillus diphtheriae* into a healthy patient, the usual pathological condition results, that is, the production of lesions characteristic of that disease, apparently with the production of additional toxin in the system.

THE BACTERIAL THEORY

In one of the earliest of the scientific reports on the mosaic disease of tobacco, that of Mayer ('86), bacteria were regarded as the causal agency, although no satisfactory proof was afforded.

Iwanowski ('03) describes the presence of bacteria-like as well as amoeba-like bodies within the tissues. The bacteria were described as intracellular, occurring in the vicinity of the cell wall, and extremely minute in size. Being the first to demonstrate the filterable character of the tobacco mosaic agency, it is rather interesting that he (Iwanowski, '92) ultimately concluded that bacteria were causally related to the disease. Strangely enough, he did not employ the skill in determining this point that he applied to other aspects of the problem. In recent years, in spite of the rapid advances in the culturing of bacteria, the bacterial view has gained few, if any, consistent adherents. A small nitrate-reducing streptococcus found in mosaic-affected tobacco is briefly referred to by Boncquet ('16, '17). Bacteria-like bodies were also identified by Dickson ('22), and he endeavored to culture the organism. By his method, bits of affected leaves were cut out, and after short disinfection intervals in alcohol and mercuric bichloride, crushed in tubes of bouillon. As clearly recognized by him there could be no certainty that surface organisms were killed. Dickson, however, secured infection by inoculation from these tubes after clouding occurred. Similar results, as he indicates, might be obtained by this method, in view of the amount of the agency originally inserted, whatever the nature of the infective particles, and the clouding with bacteria may have been entirely from secondary or surface forms.

It is rather significant that there are so few adherents of the bacterial nature of mosaic diseases. On the other hand, there is no great amount of published evidence against the bacterial viewpoint. This is certainly not wholly due to lack of effort to find bacteria, but rather to two facts: first, in at least half a dozen cases personally known to the writers, where extensive studies were made, the negative evidence was considered of too little consequence for publication; and second, acceptance of the filterable organism view tended to discourage search for bacteria. The bacterial view may be regarded at present as wholly unsustained.

THE VIRUS OR FILTERABLE VIRUS THEORY

The connotation of "virus" is fairly definite, inasmuch as the term is now generally restricted to homologize with a filterable agency of disease. In the present paper we shall use the term virus in the general sense just referred to. The ability of the agency or "organism" to pass through the pores of a standard Berkefeld or Chamberland filter is the usual criterion. Some would undoubtedly define a virus as an ultramicroscopic organism, probably of bacterial nature. This, however, was not the final view of Beijerinck ('99a) who postulated a "contagium vivum fluidum" as the cause of the mosaic disease of tobacco. While his agar filtration studies may now be regarded as inadequate, the capacity of the infectious agencies of several mosaic diseases to pass through certain standard filters under certain conditions has now been demonstrated by Iwanowski ('92), Beijerinck ('99), Allard ('16), Doolittle ('20), Duggar and Karrer ('21), and others. After summarizing an interesting study of the properties of the virus of tobacco mosaic Allard ('16a) is convinced that "there is every reason to believe that it is an ultramicroscopic parasite of some kind." If this evidence of the "filterable" nature of the disease is admitted, it would bring the causal agency into a class possibly composed of a rather miscellaneous group of bodies, since there are well-known analogies in the agents of animal disease. The "virus" view in one form or another has been widely held, but the favorite idea has been an ultramicroscopic organism.

THE AMOEBA OR PROTOZOAN THEORY

It has been pointed out that both Iwanowski and Hunger drew attention to the presence in mosaic plants of bodies which they interpreted as amoeba-like. These, however, were of relatively infrequent occurrence. More recently, in a study of the mosaic disease of sugar cane in the tropics, Matz ('19) has found certain cells of the affected tissue filled with a granular matter. Using reliable cytological methods, Kunkel ('21) confirms the presence of such cells in a mosaic disease of corn, to which, however, he seems very justly to attach no significance. Kunkel does find in the cells of diseased corn peculiar plasma-like bodies in the vicinity of the nucleus. He has also been able to distinguish similar structures in the "diseased" areas of *Hippeastrum* (Kunkel, '22) also affected with a mosaic disease. This is a very clean-cut demonstration of a plasma-like body, but whether or not it may be a modified cell structure, a pathological by-product, a colony of granular bodies, or something else, is not yet clear, nor is its significance in relation to the "mosaic" disease of these monocotyledons known.

A study of tobacco mosaic in Sumatra by Palm ('22) brings casual reference to some abnormal structures. He refers very briefly to cytological work on this mosaic and mentions the occurrence of corpuscular bodies more opaque than the general protoplasm. Likewise, he notes the occurrence of "a second foreign cell element, consisting of extraordinary, small granules." With this hazy evidence he proceeds to relate the bodies to the "so-called corpuscles of Gardner" and concludes that a "Strongyloplasma" species must be considered as the cause of the disease. Indeed, he designates the "organism" *Strongyloplasma Iwanowskii*, promising a more extended publication.

The sensation of the joint meeting of the Botanical Society of America and the American Phytopathological Society at Boston in December, 1922, was a report by Nelson on "The Occurrence of Protozoa in Plants Affected with Mosaic and Related Diseases." The stage was well set for such an announcement. The titles of several papers arranged for that same meeting indicated the finding of unusual structures in the cells of several plants affected with mosaic-like diseases. The careful work of Kunkel ('21, '22) referred to earlier in this paper; the observations of Matz ('19), Palm ('22), and Dickson ('22); the attention recently bestowed upon the existence in the spurge and milkweed families (Lafont, '10; França, '20; and Mesnil, '21) and other dicotyledons (Franchini, '22, '22 a-g) of flagellates normal to the latex tubes;—these considerations all served to establish an atmosphere on that stage exceedingly favorable or impressionable in respect to protozoology. Under such conditions Nelson described or presented upon the screen in the form of photomicrographs evidence for the existence in bean mosaic of 6 principal forms or types of a protozoan organism alleged to occur in the phloem of a diseased plant. It is impracticable here to take the time to indicate the characteristics of most of these types of flagellates described. The paper has since appeared as a technical bulletin of the

Michigan Agricultural Experiment Station (Nelson, '22). Besides the bean mosaic, similar diseases of clover and tomato, and the leaf roll of potato are reported replete with protozoans. It may not have appeared remarkable at the time the paper was presented but it is significant now that Nelson gave no picture of the conditions in the comparable cells of healthy plants. In two places in the printed paper (Nelson, '22) he refers to healthy tissue, one reference being to the potato, where he says, in part, "No organisms have been found in the sieve tubes of these plants in all the slides examined;" whereas in a general discussion of relationship he affirms that "the finding of definite protozoan organisms in constant association with mosaic plants and their absence from healthy ones indicate that they are probably the factor so long sought as the cause of these diseases."

We have endeavored to supplement this work with an elaborate cytological study of healthy and diseased tobacco and tomato tissue, healthy bean tissue, and healthy cucurbit tissue. In the Solanaceous plants we find in the phloem and in other elongated cells of perfectly healthy individuals precisely the same bodies that are found in diseased tobacco and tomato plants. The number of cells with such inclusions is not great. The most characteristic of these bodies are often sinuous, or screw-like, also of other types. They are usually homogeneous and often appear to be waxy in nature. Some supernumerary nuclei or cytoplasmic aggregates are also observed, and the remains of plastids may be associated with these. We have not studied diseased bean tissue, but in healthy tissue the long cylindrical, ovoidal, or elliptical bodies are generally homogeneous in character, centrally disposed, and frequently associated with cytoplasmic strands, the latter giving the appearance of one or more flagellae at the ends. It seems apparent that these particular bodies are those that have been described by Strasburger in normal sieve tissue. In certain cucurbits, notably in *Chayote edule*, disintegrating plastids in cells undergoing rapid elongation present the appearance of organisms of various types, all more or less nodose. It seems unnecessary to describe these bodies further in the present connection. It is clear that the peculiar structures portrayed by Nelson are all to be found, but our claim is that essentially all of these may be paralleled in perfectly healthy tissue. Moreover, the relations of these bodies seem in no way to suggest flagellates that may be normal to the tissues, whether diseased or healthy. From our studies we are convinced that these "flagellates" are made up of several factors, and while we have not attempted a careful micro-chemical examination, nor made a complete study of the developing tissues, such possibilities as the following may be noted: elongated masses of gummy material long known to be characteristic of certain sieve tissues; cytoplasmic aggregations or areas of contraction possibly associated with disintegrating plastids; elongate, accessory, and perhaps disintegrating nuclei; and homogeneous aggregates of unknown origin, possibly of waxy nature.

The importance of the problem justifies the feeling that a complete reinvestigation of these cell phenomena is being pursued by many others, and that out of it may come some compensating observations that will throw light rather than shadow on the nature of the mosaic diseases.[1]

ULTRAFILTRATION EXPERIMENTS

The fact so frequently confirmed that the agency of mosaic disease passes freely through the pores of the average Berkefeld or Chamberland filter did not establish, prior to 1921, the size of the infective agency further than to indicate that it is considerably less than that of the usual plant or animal pathogen. It sufficed merely to relate the agency to filterable organisms. In order ultimately to determine more accurately the relations of this agency it seemed essential to make a detailed study of its size relations. This was done by the present writers (Duggar and Karrer, '21), and reference to this work is a necessary preliminary to the further results which will be reported and to the theoretical considerations which we wish to present.

The work referred to consisted first in securing graded series of ultrafilters, some of which should permit the infective particles to pass freely (as shown by the infectiousness of the juice) and

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[1] Since the oral presentation of this paper, much additional light has been thrown upon the distribution and relations of these abnormal bodies described by Nelson, and similar structures, in a series of papers published in Phytopathology, Vol. 13, No. 7, 1923, by the following authors: (1) Kotila and Coons; (2) Doolittle and McKinney; (3) Kofoid, Severin, and Swezy; and (4) Bailey.

others with pores or lacunae so fine as to prevent or greatly inhibit the passage of such particles. A second phase of the work involved a careful technique in the use of the ultrafilters. A third phase required the inoculation of healthy plants with the various filtrates obtained in order to determine the percentage of dilution of the particles, if possible. Finally, some method of standardization of the filters was necessary whereby their capacity to permit or prevent the passage of particles might be related to colloidal particles of known, or approximately known, sizes. It will be unnecessary to go into the details of these experiments. Two aspects of the results require emphasis. It was possible to find a filter, in this case a cylindrical, porcelain atmometer cup, which in a given interval of time, at a given pressure, and at the reaction of the diseased tobacco juice, permitted only a relatively small number of the infectious particles to pass through. Considerable dilution of the juice from the standpoint of these particles was therefore effected. This was shown by a reduction in the incidence of infection from 90–100 per cent in the usual controls to 5–20 per cent in the case of the porcelain filter only partially permeable to the infective particles.

Standardization of the filters was accomplished by the use of hydrophilic colloids of biological origin. These were selected in preference to sols of inorganic origin, such as gold sols, because of possible greater complications (when employing the latter) arising from electrical relations. The series of organic compounds employed included casein, gelatin suspensions, lactalbumin, hemoglobin, and dextrin. Fortunately, this series sufficed. The results indicated that the hemoglobin content of a standard hemoglobin solution prepared from fresh ox blood was diluted to a very considerable degree in passing through the same filter which obstructed to a large degree the passage of the infective particles. In experimenting further with substances on either side of hemoglobin, in reference to size range, it was clear that from such filtration experiments the deduction must be made that the infective particles of mosaic disease approximate in size those of a fresh 1 per cent hemoglobin solution.

The best data on the size relations of hemoglobin particles indicated a diameter of approximately 30 $\mu\mu$. It is presumable that we are dealing in the case of a colloidal solution with particles and not with molecules. This particle size is to be compared with an average short diameter of about 1000 $\mu\mu$ for many pathogens.

If we are dealing with an organism, that is, an organized ultra-microscopic individual of approximately 30 $\mu\mu$ in diameter, its life relations must be very different from those of an organism whose volume relations are to this as 37000 to 1 or about 1,000,000 to 26. This would be the relation between the average bacterial plant pathogen and the mosaic virus. Assuming a complex organization, many theoretical questions would arise for consideration. Among these might be mentioned perhaps above all that of the surface tension conditions in such a structure, also the possibility of organization at all (membrane existence, etc.) as now comprehended.

The filtration work has been repeated with scrupulous care and it has led to results similar to those above described,—invariably pointing to an infectious particle with a size approximating that of fresh 1 per cent hemoglobin. A question which then forces itself upon the attention is: What is the peculiar nature of such a particle? To arrive at a tentative answer to this question, it would be necessary to consider all known properties of the agency, to analyze the data already carefully worked out, to plan many experiments of an entirely new type with a view to determining the behavior of the body concerned, and to contrast the inception and course of the mosaic disease or related phenomena of chlorosis in other plants. As far as possible it would be essential to examine also any possible relations of the viruses of animal diseases that may assist in one or more general interpretations.

Under the most favorable growing conditions the period of incubation of the tobacco mosaic is from 10 days to 2 weeks. By period of incubation, in this connection, is meant the time required for the development in the young leaves of the infected plant of unmistakable symptoms of mottling. In this interval of time the infective agency is widely distributed in the plant. It is not confined to the leaves (young) capable of exhibiting pronounced or favorable mosaicing, but may be found in older leaves, roots, etc. It has in reality a phenomenal power of "migration" from cell to cell,—a power none the less pronounced even if the vascular system should be shown to constitute one of the paths of this migration. Moreover, the power of migration is not a matter which may be easily determined.

The rapid and almost complete distribution of the organism in the tissues accords well perhaps with a body minute in size and attenuate in form, or else a body so fluid in character as to be capable of assuming an extremely attenuate form. A living structure so pliable and attenuate might be expected to be sensitive to reagents and conditions. To a considerable degree this sensitiveness is not true of the virus of mosaic. In our experiments it resisted the usual procedure of dehydration by means of acetone and alcohol. Modifications of the Buchner method for the extraction of enzymes (zymase) from yeast cells were applied to a pulp of fresh, diseased, leaf tissue ground with very fine quartz sand, 5 parts of the former to 1 of the latter. Three series of experiments were arranged. In the first, the pulp was treated with full-strength acetone. There were 2 treatments, each of 3 minutes, the tissue being drained after the first addition of acetone and then fresh acetone applied for an equal interval. Finally, the material was dried as promptly as possible under an electric fan. In the second case the dehydration treatment consisted in the addition of 95 per cent alcohol for a 3-minute interval, followed by pure acetone, and finally dried, as above. In the third case the treatment was 95 per cent alcohol, followed, in the same intervals as before, with 98 per cent alcohol, and finally dried. After 3 days these residues were extracted with water, each for 1 hour, using about 10 parts of water to 1 of the dried material. After the filtration of each extract through cotton, 20 plants were inoculated with each extract, and suitable (20) controls were maintained. These plants were under favorable growth conditions, and they exhibited the symptoms of disease promptly. At the end of 19 days, 1 healthy plant only remained in each set. All uninoculated controls remained healthy, and the incidence of disease in the control which was inoculated with diseased juice was likewise 19.

When, however, the amount of alcohol and acetone was greatly increased in relation to the bulk of material used (approximately 200 times as much), the incidence of infection was low, showing that the infective particles do not withstand complete dehydration.

A study of the effects of longer exposure to various grades of alcohol has been carried out at some length by Allard ('16a), who found that relatively speaking the infective properties are quickly destroyed by the higher strengths of ethyl alcohol. He indicates destruction by 80 per cent alcohol in 30 minutes. On the other hand, in later work, he (Allard, '18) has shown some striking resistance of the infective agency to the weaker grades of alcohol. We may note a few instances. Kept in 25 per cent alcohol 34 days and then inoculated into the host, 7 out of 10 plants developed mosaic; this, however, is obviously exceptional, since in another test the virus kept in 25 per cent alcohol 199 days yielded 7 out of 10 diseased plants; in 50 per cent alcohol after 40 days no disease was induced; in 50 per cent alcohol after 35 days 4 out of 10 plants became diseased. With another sample of the virus in 50 per cent alcohol for about 5 days only 2 plants were diseased after inoculation from this material.

From a comparison with active cells in the vegetative condition it will be seen that these results indicate a high degree of resistance since the average bacterial cell may be injured after 24 hours by a concentration of from 5 to 10 per cent alcohol. Moreover, the yeast cell, which shows a specific tolerance of alcohol concentration, is itself injuriously affected by more than about 15 per cent alcohol. If the comparison is made with the tolerance of the spores of certain species of bacteria, we shall find that the infective particles of mosaic are less resistant. Feeling that it was unwise to accept some of the data which have been published on this point, we made a study of the tolerance of the spores and vegetative cells of the hay bacillus, *Bacillus subtilis*.

In the duplication of this work the senior author was assisted by Dr. H. R. Rosen. One cubic centimeter of a dense infusion of this organism was placed in a series of alcohols diluted with a decoction of tobacco juice so as to get respectively 10, 20, 30, 40, and 50 per cent alcohol, and similar concentrations of acetone. At intervals up to 10 days, streak cultures from these concentrations of the disinfectant yielded in each case continuous growth of the organism. Similar cultures were made with a young culture of the bacillus in which, of course, there were relatively few cells in the spore condition. In the latter case very few colonies appeared after the third day. In a repetition and amplification of this work, the tobacco extract was not employed, and the organism was suspended in concentrations of alcohol, as follows: 10, 20, 40, 60, and 100 per cent, with controls in bouillon and in distilled water. From all those cases in which the spore suspension was employed, a profuse growth was obtained on every streak culture from 10 to 99 per cent alcohol, with apparently no lessening of the intensity of growth as between 10 per cent and 99 per cent. In the case of the acetone-treated material, profuse growth was attained at 10, 30, and 60 per cent acetone with some indication of less intense growth in absolute acetone.

Following the above observations, made the third day, it was determined to make isolation cultures after the tenth day, and these were accordingly arranged and a careful count made as the colonies appeared. There was a progressive diminution in the number of cells alive from the 20 per cent to the 99 per cent alcohol. For that whole interval, however, this decrease amounted to only about nine-tenths of the organisms present in the infusion. There was scarcely any diminution as between 10 per cent acetone and 60 per cent, but in absolute acetone the number of organisms was considerably reduced. These data confirm the statement previously made to the effect that the virus of mosaic is less resistant than certain spore forms of the bacteria. This, however, is not surprising, for whatever may be the nature of the virus, many colloids lose to a considerable degree their hydrophilic character when treated with strong alcohol. The point of interest, therefore, is more particularly the nature of the bacterial spore which permits survival in the high concentrations discussed, a problem rather apart from our specific investigation.

EFFECT OF GRINDING ON THE INFECTIVITY OF THE TOBACCO VIRUS

Inasmuch as the thermal tolerance and the resistance toward dehydrating and disinfecting agents, while suggestive, did not seem to set off this virus as possessing properties peculiarly distinctive, it seemed particularly desirable, in view of the size relations, to determine the influence of long-continued grinding under conditions which are generally effective in disrupting living cells. A final series of experiments in this field will be sufficient to indicate the relations encountered. It should be indicated, however, that this series is in accurate accord with less extensive work previously undertaken to determine the same point. The grinding was carried out in an agate mortar with motor-driven, excentrically arranged pestle, the usual device employed in grinding bacterial cultures. Equal amounts by weight of fresh leaf material and diatomaceous earth were used.

INOCULATION EXPERIMENTS WITH FINELY GROUND MATERIAL FROM DISEASED TOBACCO LEAVES. INTERVAL, 3 WEEKS

Nature of inoculum	Total diseased after 4 weeks (ten plants inoculated)
Ground 3 hours	8 plants diseased
Ground 9 hours	6 plants diseased
Control, no inoculation	None diseased
Control, fresh dis'd. juice	7 diseased

While there has been some inconsistency in the data from other grinding experiments they point in general to one conclusion, namely, that the virus is highly resistant to protracted grinding with diatomaceous earth when the virus is ground with fresh leaf pulp. It is less resistant when filtered through porous cups, then mixed with diatomaceous earth, and ground for 9 hours. The presence of leaf material acts to prevent the greater injury. In order that these experiments may be significant it is necessary to compare the grinding of the tobacco virus with that of a species of bacteria.

For this purpose also we have employed *Bacillus subtilis* in the spore condition. Two cc. of a 22-day-old culture in bouillon (rich in spores) were thoroughly mixed with 2 gms. sterile diatomaceous earth in a petri dish. This mixture was then dried, being protected during drying by sterile paper bags. It was then subjected to grinding for the same intervals as previously employed, namely 3, 6, and 9 hours. All possible care was taken to prevent contamination of the material, but some sporadic contamination was unavoidable. As the grinding progressed, a sample was taken after each interval, the sample being removed from directly beneath the pestle. This was placed in a sterile weighing flask and weighed. One-tenth gm. of the sample was diluted to 9 cc., giving a dilution of 1:100. From this, other dilutions up to 1 to 10^6 were made, and poured plates were arranged from these dilutions. A sample from the original bouillon culture mixed in the same proportion with the diatomaceous earth, without grinding, was plated out at similar concentrations. Duplicate cultures were made in every case. The result of these experiments indicated that even after grinding the spores of *Bacillus subtilis* for only 3 hours very few remained viable, an average of 32 per culture at a dilution of 1:100, at which dilution the control showed innumerable colonies. After 6 hours of grinding the viable spores averaged 2 per culture, and no greater dilution yielded any colonies whatsoever. After 9 hours a single colony appeared at a dilution of 1:100, and no colonies at greater dilutions. Grinding was therefore thoroughly efficient in killing the spores of bacteria.

We have carried out a variety of experiments on temperature relations, the effects of disinfectants, the action of light, etc., without securing any results that indicate unusual peculiarities of the mosaic. *In vitro* studies of the mosaic agency have likewise failed thus far to give any evidence of change in the culture solutions indicative of the activity of living organisms. In addition to these lines of research we have also undertaken extensive experiments, beginning in the winter of 1921–22, in the filtration of bacteria, with the idea of determining the capacity of such organisms to pass filters when apparently the spore or cell sizes were greater than the diameters of the pores, or lacunae, of the filters employed. These experiments have yielded results of striking interest, and in time they will be published separately. As bearing on the particular problem in hand, however, no application of the investigation seems possible, both because of the inactivity of the mosaic "virus" and the lack of evidence of any stage of the latter of microscopic dimensions.

In endeavoring to arrive at something more concrete than the mere name "virus" to explain the general nature of the mosaic disease agency, we need to recall many facts bearing upon somewhat related phenomena. From the investigations of Lindstrom

('18) the inheritance of a number of chlorophyll types is shown to be strictly Mendelian. These types involve various degrees of striping and cases in which the chlorophyll is almost or entirely suppressed, with the production of white, virescent, or yellow seedlings. Passing from these normally inherited color characteristics to those which are infectious, such cases as those of the variegated *Abutilon* and the striped *Ligustrum*, worked upon by Baur ('06, '07, '08), come up for consideration. In these cases, it will be recalled, there is a characteristic pattern of color, but there is no noticeable tissue modification. Transmission is by grafting only.

The types just discussed, without graft-infection experiments to demonstrate their peculiarities, would be considered "normal" variations. The infectiousness, however, is precisely that which was found by Erwin Smith to prevail in peach yellows. "Peach yellows" is, in part, a chlorotic disease, but it gradually leads to severe injury and ultimately death of the peach tree. The disease is not transmitted by pollen nor, so far as known, by seed, but a diseased scion will convey the disease in time to a healthy stock. This disease has been considered by many to possess a highly infectious nature, but of this infectious character the senior writer has been wholly unable to find any authentic proof. Statements indicating that it may "sweep an orchard in a few years," when followed up are found to be equally as well explained by the possibility that all the stock came from a single nursery at the same time. Scions from the same tree may have been employed. This disease, moreover, is rather closely localized in a narrow climatic zone. The claims of sporadic appearance of the disease in regions far south of Michigan and Delaware have in very few, if any, cases been adequately verified, especially since the water-shoots arising in clusters from severely headed-back or winter-injured trees possess many characteristics of yellows, clearly recognized, however, as water-shoots by the expert.

One should include in the graft-transmissible forms reference to the recent work of Blakeslee ('21), in which a graft-infectious disease of *Datura* resembling a vegetative mutation is discussed and its behavior in heredity clearly set forth. This disease has been known as the *Quercina* form. It is not artificially transferable except by grafting, but certain other species of *Solanaceae* are susceptible through grafting. It is transmitted by seed to about 79 per cent of the offspring when pollinated with normal plants.

The case of the curly-top of sugar-beets, which is generally assumed to be related to mosaic disease, is peculiar in that no infection by diseased juice can occur until the juice has passed into the body of *Eutettix tenella*, in which it must remain a definite time interval, or incubation period, before being infectious to beets.

In this case not even grafting has been successful according to the more recent reports. In still another category with respect to infectiousness may be included the case of mosaic in sugar-cane, poke-weed, and other plants in which insect transfer is the more effective method yet found.

The well-known cases in tobacco, bean, cucurbit, and other plants wherein the transfer of juice from diseased to healthy plants, whether by aphids or by needle prick, is sufficient to reproduce the disease,—these are more closely related, as to infectiousness, to ordinary bacterial or other parasitic diseases. Detailed experiments by us confirm the view that the virus of tobacco and of related mosaic diseases do not pass readily, if at all, through uninjured surfaces. We have tested this by spraying the diseased juice on the leaves, also by placing the diseased juice in glass cells sealed to leaf surfaces for 24 hours or more. Under such conditions the virus is practically inert.

It is suggestive that in the tobacco mosaic, the tomato mosaic, and many others, the gametes do not seem to possess the virus; at least the embryo arising from the fused gametes is not diseased, while the seed-coats are. It is conceivable that the reduction division is concerned in the elimination of the disease, a possibility which, if established, would be significant. The case of the bean is, however, an apparent exception, though the possibility of infection after early embryonic development is not excluded.

Time prevents a more complete discussion of the bearing of these facts, but the trend of the evidence seems to indicate that we have here a group of viruses which, apart from the cell, are as inactive as any colloidal particle lacking that correlated organization which is characteristic of cell life. Within the cell such a virus possesses unusual activity, obviously. So far as resistance to environmental conditions is concerned, we have to admit frankly that there may be no great difference between a living cell, and enzyme, and many types of biocolloids, but, on the whole, the mosaic virus behaves as if it were a biocolloid, yet one endowed with the power of reproduction. Now it has been frequently suggested in the literature that all these discussions as to the nature of a virus are unnecessary, since we may just as well take the easier, simpler view, and call a virus an ultramicroscopic organism. The facts are just a little out of line, if viewed in their broadest aspects; and the fascination is to go on and perhaps ultimately get a satisfactory explanation, or arrive at what may be an acceptable theory.

We cannot forget that important contributions have been made almost within the year. The d'Herelle phenomenon is itself a remarkable discovery. Here is a filterable body—call it what you like—appearing in the excretions of dysentery, which, placed in contact with the bacterial culture, is lethal to the culture; and at the same time the body propagates itself.

Again, if all viruses are minute bacteria, why are there no analogues of such microorganisms as saprophytes? Why are there none in butter, in milk, in soil, in fermentation phenomena of one type or another? While some "indications" of the existence of filterable organisms in such environments have been reported, it must be admitted that all changes in such substrates have been related to organisms that are not ultramicroscopic, and no such parallel in nature has been clearly demonstrated so far as I am aware. There are, of course, many diseases induced by extremely small microorganisms but the question is: Have we not already reached the point where our technique may always make evident some stage of such organisms? Are any truly ultramicroscopic organisms culturable? With agencies of the mosaic virus type we have made no progress, possibly because progress is not attainable by culture methods and by microscopic vision.

In respect to size relations some pertinent questions also arise. Is it, for example, possible that a protoplasmic particle may be as small, in small diameter, as a hemoglobin particle, remembering that the former must carry the properties of surface and central plasma and of nucleoplasm—indeed of all the characteristics of an individual ? This particular question does not seem to us to be affected by any consideration of the magnitude of the long diameter of the individual. Such an individual could not presumably penetrate cell walls, and its rapid spread through the tissues would be dependent upon bridging protoplasmic fibrils between the cells.

If one is compelled to admit the existence of an organism of the size relations above referred to, it would seem necessary with the data at hand to conceive of it as a flagellum-like creature with perhaps a temporary hook-up of molecules or colloidal particles, conceivably with no true bordering membrane and no restricted endometabolism. The supposition that the organism might be of an extremely fluid nature would perhaps be equally unsatisfying.

Taking into consideration all the facts, we cannot avoid the impression, tentatively, that the causal agency in mosaic disease may be, in any particular case, a sometime product of the host cell; not a simple product such as an enzyme, but a particle of chromatin or of some structure with a definite heredity, a gene perhaps, that has, so to speak, revolted from the shackles of coordination, and being endowed with a capacity to reproduce itself, continues to produce disturbance and "stimulation" in its path, but its path is only the living cell.

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